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Melanoma Cells Control HA Synthesis in Peritumoral Fibroblasts via PDGF-AA and PDGF-CC: Impact on Melanoma Cell Proliferation

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The microenvironment surrounding tumors has an important role in tumor progression. Fibroblasts (Fbs) of the tumor stroma receive signals from tumors and transform the extracellular matrix, thus supporting tumor growth, motility, and metastasis. The matrix component hyaluronan (HA) has a pivotal role in tumor progression. Here we analyzed the cell populations that synthesize HA in human malignant melanoma (MM) cells and studied the regulatory network between MM cells and stromal Fbs controlling HA synthesis. Tissue analysis indicated that Fbs are the main source of HA in the stroma of melanoma, whereas MM themselves synthesize only minute amounts of HA. *In vitro*, Fb-derived HA is mainly produced by hyaluronan synthase 2 (HAS2) and enhances proliferation of MM. Proteins secreted by MM can further increase HA synthesis in Fbs in a phosphatidylinositol 3/mitogen-activated protein-kinase-dependent manner. Melanoma cell–derived platelet-derived growth factor (PDGF)-AA and PDGF-CC were identified as major mediators that signal through PDGFR-α and thus induce HAS2-mediated HA synthesis in Fbs. In conclusion, we have identified a complex interaction of MM with its surrounding microenvironment by demonstrating that MM by the release of PDGF-AA and PDGF-CC upregulate HA synthesis in Fbs, which in turn stimulates MM proliferation in a paracrine manner.

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INTRODUCTION

Tumor–stroma interactions have a pivotal role in cancer biology. The tissue microenvironment surrounding the tumor, especially cancer-associated fibroblasts (Fbs) in the stroma of solid tumors, supports tumor growth, motility, and metastasis (Kalluri and Zeisberg, 2006; Ostman and Augsten, 2009). Malignant melanoma cells (MM) activate Fbs to release increased amounts of metalloproteinases, glycosaminoglycans, and cytokines, thereby transforming the extracellular matrix to support tumor progression (Wandel *et al.*, 2000; Loffek *et al.*, 2005; Zigrino *et al.*, 2005, 2009).

The glycosaminoglycan hyaluronan (HA), a high molecular unbranched polymer of repeating disaccharides of glucuronic acid and *N*-acetylglucosamine, seems to have a crucial role in tumor growth and metastasis (Toole, 2002). HA is synthesized by three different HA synthases (HASs), namely HAS1, HAS2, and HAS3, which are located at the inner side of the plasma membrane (Weigel *et al.*, 1997; Tammi *et al.*, 2002). The synthases differ in their enzymatic properties concerning HA elongation rate, enzymatic activity, and K_m-value for the substrates (Itano *et al.*, 1999). Enzymatic degradation of HA is performed by hyaluronidases when high-molecular-weight HA is cleaved by Hyal-2 and the resulting 20 kDa fragments are further processed intralysosomally by hyaluronidase-1 (Stern, 2003; Stern *et al.*, 2006). The importance of HA for tumor progression has been demonstrated for many solid tumors, including breast cancer (Auvinen *et al.*, 1997; Bose and Masellis, 2005), colon carcinoma (Misra *et al.*, 2008), and ovarian cancer (Tammi *et al.*, 2008). Tumors with an HA-rich stroma have a growth advantage compared with those without (Tammi *et al.*, 2008). HA promotes angiogenesis (Itano *et al.*, 2008), modulates antitumor response by immobilization of inflammatory cells (Day and de la Motte, 2005; Itano and Kimata, 2008), supports tumor cell motility (Afify *et al.*, 2009), and enhances proliferation (Ahrens *et al.*, 2001; Itano *et al.*, 2008).

It has been shown before that MM supernatants stimulate HA synthesis in dermal Fbs (Knudson *et al.*, 1984; Merrilees and Finlay, 1985; Godden *et al.*, 1999; Edward, 2001) and various MM-derived paracrine mediators have been analyzed. However, the mechanisms of HA synthesis and the contribution of the enzymes of the HA metabolism are still unknown. In this paper we compared the HA metabolism in

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Abbreviations: CM, conditioned medium; Fb, fibroblast; HA, hyaluronan; HAS, HA synthase; MM, malignant melanoma cells; MM-CM, malignant melanoma cell–conditioned medium

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human Fbs and MM and analyzed cell lines with regard to their expression of individual HAS enzymes and their impact on HA release by MM-exposed Fbs. We show that Fbs are the major source of HA. Moreover, we confirmed that HAS1 and HAS2 in Fbs are regulated by MM-derived mediators and demonstrate that MM-derived PDGF-AA and PDGF-CC stimulate HAS2 expression in Fbs via PDGFR-α and thus augment HA deposition by Fbs, thus substantiating previous data from Godden *et al.* (1999). The HA synthesized by activated dermal Fbs enhanced proliferation of MM *in vitro*, suggesting that also *in vivo* stromal HA deposited on paracrine control of MM-derived soluble mediators might contribute to melanoma progression.

RESULTS

Stromal Fbs surrounding melanomas are the main producers of HA *in vivo* and *in vitro*

To identify HA-synthesizing cell types in MM, immunoaffinity stainings with biotinylated HA binding protein (HAbP) of melanoma specimen were performed. HA was detected predominantly in the tumor stroma (Figure 1a and b). Using double staining with an Fb-specific antibody (Saalbach *et al.*, 1997) we found predominant colocalization of HA and Fbs (Figure 1c). MM themselves clearly showed weaker HA staining (Figure 1a–d).

In vitro, basal HA synthesis was analyzed in cultured dermal Fbs and MM by ELISA. Fbs released over a thousand times more HA into the culture supernatants compared with identical numbers of MM (Figure 1e). In accordance, HAS2–mRNA was significantly stronger expressed in Fbs than in MM as detected by quantitative reverse transcription (RT)-PCR (Figure 1f). HAS2 represents the main HA-synthesizing enzyme in resting Fbs (Averbeck *et al.*, 2007). HAS1– and HAS3–mRNA were only expressed at low levels in all cell types (data not shown).

MM proliferation is increased by Fb-conditioned culture supernatants, depending on Fb-derived HA and soluble mediators from MM further stimulate HA-synthesis in Fbs

In medium transfer experiments we tested whether MM benefit from Fb-derived HA and observed a significantly increased proliferation rate of MM growing in Fb-conditioned medium (Fb-CM; Figure 2a). When HA synthesis in Fbs was inhibited by 70–90% (Figure 2b) by silencing of HAS2, the proliferation rate of MM decreased markedly (Figure 2a), thus suggesting proproliferative effects of Fb-derived HA.

Next we studied the influence of MM-derived soluble factors on the HA synthesis in Fbs. Fbs were incubated with CM (melanoma cell–conditioned medium, MM-CM) of different melanoma cell lines. *Bro*- and *HT144*-CM induced a significant increase of HAS1– and HAS2–mRNA expression in Fbs compared with control, whereas *A375*-CM did not (Figure 2c and d). No changes in the HAS3–mRNA expression were induced by any of the CM (data not shown). Consequently, HA release by Fbs was significantly increased after 24 hours of stimulation with *Bro*- or *HT144*-CM and not affected significantly by *A375*-CM (Figure 2e), suggesting a lower inductive capacity of the latter cell line. Incubation of MM with Fb-CM did not modify low HA synthesis of MM (data not shown).

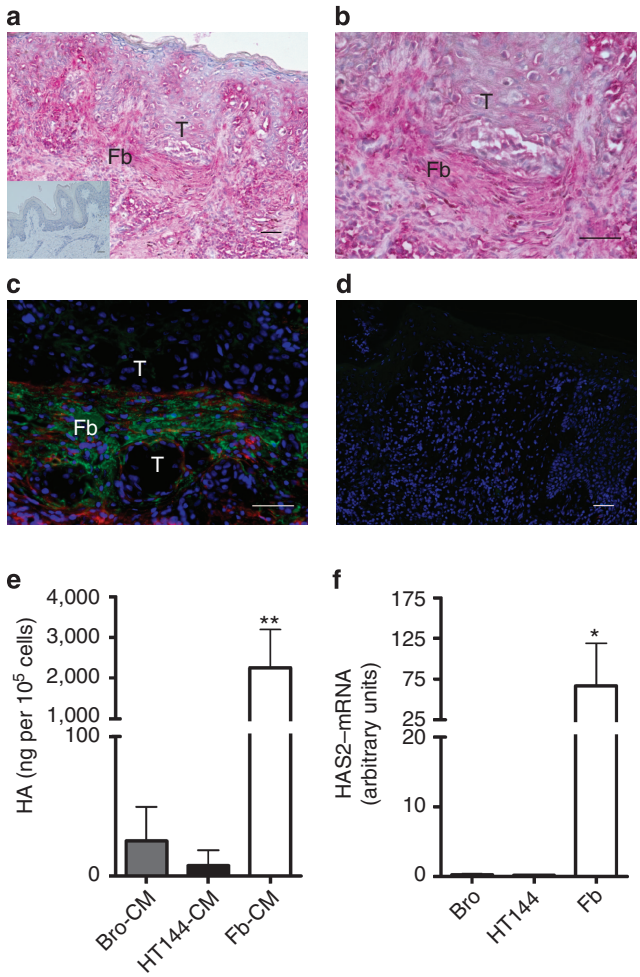

Figure 1. Hyaluronan (HA) and hyaluronan synthase 2 (HAS2)–mRNA are predominantly expressed by stroma cells *in vivo* and *in vitro*. HA in malignant melanoma (MM) is deposited mainly in the fibroblast-rich stroma. (**a**–**d**) A superficially spreading melanoma (Clark III, Breslow 1.2 mm) is shown. (**a**,**b**) HA was detected with biotinylated HA binding protein (HAbP) and FastRed development giving purple-red staining for HA. Counterstain was performed with hematoxylin. The tumor cells (T) express less HA compared with the surrounding stromal fibroblasts (Fbs). Inserted picture in **a**: tissue staining after pre-treatment with hyaluronidase. (**c**) Fluorescence image of the same biopsy with HAbP-biotin detected by streptavidin-488 and anti-CD90 detected by goat-anti mouse-Cy3. The nuclei were counterstained with 4,6-diamidino-2-phenylindole. Again, stroma areas rich with Fbs (red) contain more HA than the tumor. (**d**) Streptavidin-488 control without HAbP and isotype control for anti-CD90. Bar = 50 μm. (**e**) *In vitro* HA is released predominantly by Fbs ($n = 4$), and (**f**) Fbs express significantly more HAS2–mRNA than MM ($n = 5$). *P*-values were calculated using unpaired *t*-test. Data are presented as mean ± SD. $^*P < 0.05$ and $^{**}P < 0.005$. CM, conditioned medium; Fb-CM, Fb-conditioned medium.

To identify melanoma-derived soluble mediators that stimulate HA synthesis in Fbs, we tested whether lactate, a known inducer of HA (Stern *et al.*, 2002; Rudrabhatla *et al.*, 2006), or soluble proteins stimulate HA production. First, lactate concentrations in supernatants of MM with different

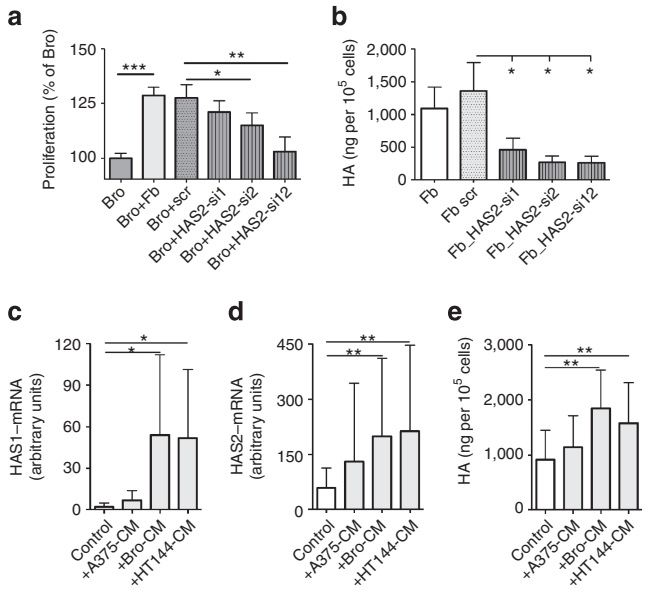

Figure 2. Fibroblast-derived hyaluronan (HA) stimulates malignant melanoma (MM) proliferation. MM-derived mediators stimulate hyaluronan synthase 2 (HAS) expression and HA synthesis in fibroblasts. (**a**) Bro proliferation was assayed in fibroblast-conditioned medium (Fb-CM) of control fibroblasts, fibroblasts transfected with scrambled control (scr), and HAS2-silenced fibroblasts ($n=4$). Proliferation of Bro cells alone was set to 100%. (**b**) The silencing of HAS2–mRNA results in significant reduction of HA content in the corresponding FB-CM ($n=8$). *P*-values were calculated using unpaired *t*-test. (**c**, **d**) Dermal fibroblasts were exposed to MM-CM. Fibroblasts were harvested after 3 hours for quantitative reverse transcription-PCR of (**c**) HAS1– and (**d**) HAS2–mRNA (A375-CM ($n=3$); Bro- and HT144-CM ($n=5$). (**e**) After 24 hours incubation with MM-CM, HA was quantified in Fb-CM ($n=7$). *P*-values were calculated using Wilcoxon signed rank-sum test. Data are presented as mean ± SD. $^*P<0.05$, $^{**}P<0.005$, and $^{***}P<0.0005$.

HA-inducing potential did not vary (Supplementary Figure S1a online), thus being excluded as potential inductor. Second, *Bro*-CM was treated with trypsin beads to degrade proteins, verified by Coomassie staining (Supplementary Figure S1b online). Fbs incubated with protein-depleted *Bro*-CM did not show increased HAS–mRNA compared with untreated *Bro*-CM (Supplementary Figure S1c and d online), suggesting that proteins might be responsible for upregulation of HA synthesis in Fbs.

Melanoma-derived TGF-β_1 has significant effects on HAS1–mRNA, but not on the net HA synthesis by Fbs

Transforming growth factor (TGF)-β_1 was reported to stimulate HA synthesis in different cell types (Sugiyama *et al.*, 1998; Campo *et al.*, 2007; Dai *et al.*, 2007; Meran *et al.*, 2007). Here, recombinant TGF-β_1 enhanced HAS1– and HAS2–mRNA expression in Fbs and increased HA production by ~30% (Supplementary Figure S2a online). TGF-β_1 was released from *Bro*- and *HT144*-CM (*Bro*: $140.2 \pm 38.8\,pg\,ml^{-1}$, *HT144*: $153.9 \pm 97.8\,pg\,ml^{-1}$ per 10^5 cells), but was barely detectable in *A375*-CM ($<30\,pg\,ml^{-1}$), which is in accordance with HA-inducing properties of these cell lines. Silencing of TGF-β_1 with small interfering RNA (siRNA) in *Bro* and *HT144* melanoma cells decreased TGF-β_1 by about 90% in the resulting MM-CM (Supplementary Figure S2b online). Fbs were exposed to these TGF-β_1-reduced MM-CM. The inductive effect of MM-CM on HAS1–mRNA in Fbs was significantly diminished with TGF-β_1-reduced MM-CM compared with scrambled control (Supplementary Figure S2c online). In contrast, TGF-β_1-reduced *Bro*-CM did not affect HAS2–mRNA induction, whereas TGF-β_1-reduced *HT144*-CM partially lost its stimulatory capacity for HAS2 (Supplementary Figure S2c online). Most importantly, however, TGF-β_1-reduced MM-CM still stimulated net HA synthesis by Fbs significantly (Supplementary Figure S2d online). These data suggest that despite the influence of TGF-β_1 on HAS1 and, in part, on HAS2–mRNA in one cell line *HT144*, net HA synthesis in dermal Fbs is controlled by other MM-derived mediators in our experimental setting.

Melanoma-derived growth factors induce HAS2–mRNA and HA synthesis in Fbs by the PI3/MAP-kinase pathway

As HAS2 appears not to be regulated by TGF-β_1, we investigated other putative mediator proteins. To identify promising candidates we first analyzed the signaling pathways involved focusing on the phosphatidylinositol 3/mitogen-activated protein (PI3/MAP)-kinase pathway. Addition of PI3-kinase inhibitor LY294002 or the MEK1/2 inhibitor U0126 to Fb cultures reduced the stimulating capacity of *Bro*-CM on HAS2–mRNA in Fbs (Figure 3a and b), resulting in a clearly diminished HA release (Figure 3c and d). HAS1–mRNA expression was not affected (data not shown). This confirmed HAS2 as the major HA-synthesizing enzyme in dermal Fbs, whose expression is controlled by the PI3/MAP-kinase pathway.

Comparative analysis of growth factor expression in melanoma cell lines

To identify the soluble factors released by MM responsible for stimulating HA synthesis in Fbs in a PI3/MAP-kinase-dependent manner, we took advantage of different capacities of MM cell lines to induce HA in Fbs, i.e., strongly HA-inducing *Bro*-MM and weakly HA-inducing *A375*-MM (Figure 2e). We performed a growth factor array, comparing strongly HA-inducing *Bro*-CM with weakly HA-inducing *A375*-CM. On this array we found PDGF-AA to be the most prominent growth factor in *Bro*-CM, which was expressed about six times more compared with *A375*-CM (Figure 4a and b). As PDGFs function through the PI3/MAP-kinase pathway, and different members of the PDGF family have been suggested to induce HA synthesis (Godden *et al.*, 1999), we also analyzed PDGF-AB and PDGF-BB expression on this array. These proteins were barely detectable in both cell lines, although levels in *Bro*-CM always exceeded those in *A375*-CM (Figure 4a and b). PDGF-CC, a more recently discovered member of the PDGF family (Anderberg *et al.*, 2009), was not covered by the growth factor array. Instead, we analyzed PDGF-C–, as well as PDGF-A–, and PDGF-B–mRNA by quantitative RT-PCR, including a second strongly HA-inducing melanoma cell line *HT144*. We found PDGF-A– and PDGF-C–mRNA to be significantly higher expressed in

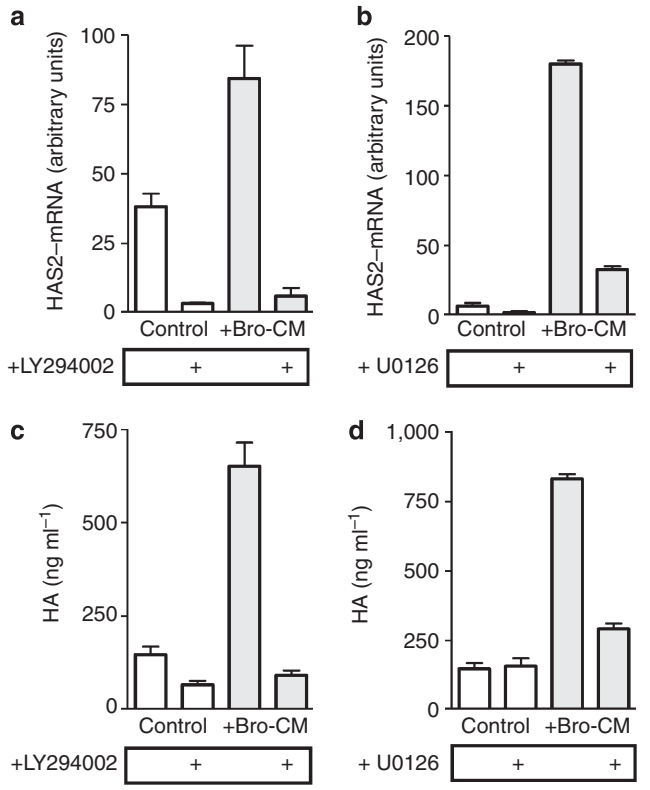

Figure 3. Inhibition of the phosphatidylinositol 3/mitogen-activated protein (PI3/MAP)-kinase pathway in fibroblasts leads to decreased stimulation of hyaluronan synthase 2 (HAS2)–mRNA and hyaluronan (HA) synthesis through malignant melanoma-conditioned medium (CM). Blocking of the growth factor signaling way was carried out using the MAP/ERK-kinase 1/2 inhibitor U0126 and the PI3-kinase inhibitor LY294002. Fibroblasts were incubated in duplicates with Bro-CM, Bro-CM + 10 μM U0126, or Bro-CM + 20 μM LY294002. Equivalent DMSO volumes were added to serum-free medium as control. (**a**, **b**) HAS2–mRNA in fibroblasts was analyzed after 3 hours incubation by quantitative reverse transcription-PCR. (**c**, **d**) HA concentration in fibroblast supernatants was measured after 24 hours by ELISA. Data are presented as mean ± SD.

HA-inducing *Bro*- and *HT144*-MM compared with *A375*-MM (Figure 4c). PDGF-B–mRNA was expressed at minimal levels in *Bro* cells only, which was in line with the array results (Figure 4c).

Melanoma-derived PDGF-AA and PDGF-CC induce HAS2–mRNA and HA synthesis in dermal Fbs

Next we silenced PDGF-A– and PDGF-C–mRNA in MM using siRNA. Target mRNAs and detectable PDGF-AA protein were reduced by 80–90% at 72 hours after transfection (Supplementary Figure S3 online). Interestingly, PDGF-A silencing led to increased PDGF-C–mRNA expression and vice versa (Supplementary Figure S3a–d online), resulting in a sustained HAS2 induction in Fbs (data not shown). Therefore, we performed PDGF-A/PDGF-C double knockdown (Supplementary Figure S3a online). PDGF-AA/CC-reduced MM-CM showed diminished ability to induce HAS2–mRNA expression in Fbs (Figure 5a), and HA concentrations in supernatants of exposed Fbs were decreased by 15–30% compared with scrambled control (Figure 5b).

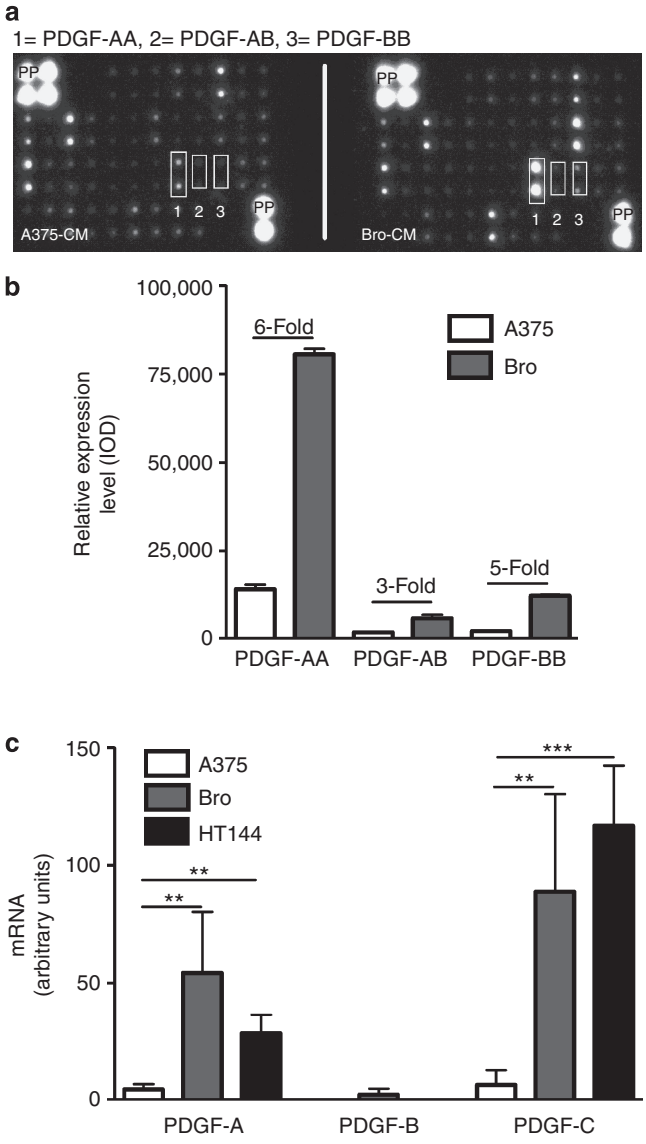

Figure 4. Comparative analysis of growth factor expression in three different malignant melanoma (MM) cell lines. (**a**) Growth factor arrays were performed to compare platelet-derived growth factor (PDGF)-AA, PDGF-AB, and PDGF-BB in weakly stimulating A375-conditioned medium (CM) and strongly stimulating Bro-CM. A typical membrane is shown with the PDGF spots indicated. PP: positive controls. (**b**) Relative protein signals on the arrays were measured as optical density of chemiluminescence ($n = 2$). (**c**) PDGF-A–, PDGF-B–, and PDGF-C–mRNA in three different MM were analyzed by quantitative reverse transcription-PCR ($n = 5$). *P*-values were calculated using the unpaired Student's *t*-test. Data are presented as mean ± SD. $^{**}P < 0.005$ and $^{***}P < 0.001$. IOD, integrated optical density.

PDGF-AA and PFGF-CC both signal through PDGFR-α (Fredriksson *et al.*, 2004; Anderberg *et al.*, 2009). Therefore, we used neutralizing antibodies against PDGFR-α on Fbs. Compared with isotype control, HAS2–mRNA induction (Figure 5c) and HA release (Figure 5d) decreased significantly in these Fbs, indicating that PDGFR-α signaling is involved in Fb HA synthesis induced by MM-CM. Interestingly, HAS1–mRNA in the exposed Fbs was not affected by these inhibitions of the PDGFR-α signaling pathway, indicating

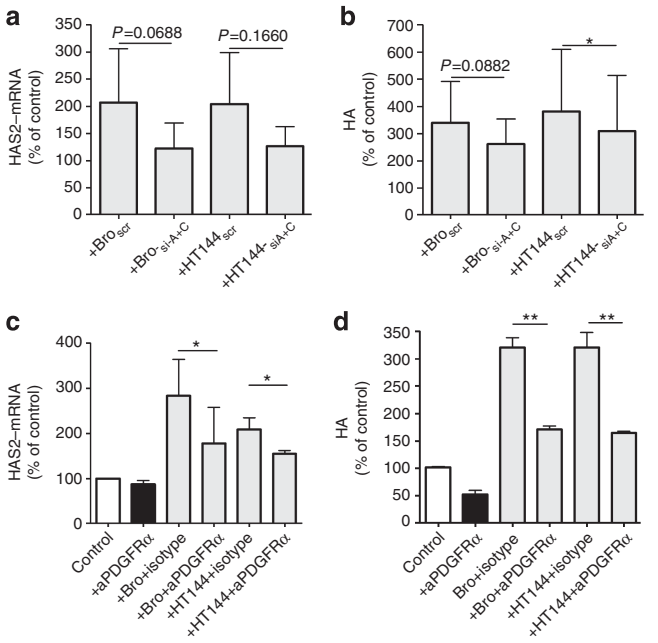

Figure 5. Silencing of platelet-derived growth factor (PDGF)-A– and PDGF-C–mRNA in malignant melanoma (MM) or blocking of PDGFR-α on fibroblasts reduces hyaluronan synthase 2 (HAS2)–mRNA induction and hyaluronan (HA) release from exposed fibroblasts. PDGF-A and PDGF-C silencing was performed in Bro- and HT144-MM (Supplementary Figure S3 online). Fibroblasts were exposed to the resulting MM-conditioned medium (CM). (**a**) HAS2–mRNA was quantified by quantitative reverse transcription (RT)-PCR after 6 hours of incubation. The results are presented as percent of serum-free control ($n=3$). (**b**) HA concentration in fibroblast supernatants was measured by ELISA after 24 hours with MM-CM ($n=3$). (**c**, **d**) Fibroblasts were pre-incubated with neutralizing antibody against PDGFR-α or mouse-isotype control before MM-CM was added. As controls, isotype incubation (set to 100%) and incubation of fibroblasts with aPDGFR-α alone (black column) were used. (**c**) HAS2–mRNA was quantified by RT-PCR after 3 hours. Results are presented as percent of serum-free control ($n=3$). (**d**) HA concentration in fibroblast supernatants was measured after 24 hours by ELISA ($n=3$). Data are presented as mean ± SD. *$P<0.05$, **$P<0.005$; Student's paired *t*-test.

that HAS2 is the main target of PDGF-AA/-CC containing MM-CM (Supplementary Figure S4 online).

DISCUSSION

Tumor–stroma interactions are essential for progression and metastasis of solid tumors (Kalluri and Zeisberg, 2006; Ostman and Augsten, 2009). Stroma cells provide a tissue scaffold allowing tumor growth and dissemination. Tumor cells modify the matrix synthesizing and degrading capacities of stroma cells by direct and paracrine interactions. Here we show that melanoma cells increase HA synthesis in dermal Fbs through soluble proteins, and that Fb-derived HA promotes MM proliferation *in vitro*.

In situ examination of MM by Karjalainen *et al.* (2000) had shown strong staining of HA in the tumor stroma, whereas the tumor itself varied in HA expression levels as found in a large cohort of stage I melanomas. In the MM biopsies analyzed here we detected stronger HAbP binding in the adjacent dermal stroma compared with the tumor, suggesting that HA was deposited by the Fbs, which were also detected in these HA-containing regions. The impact of infiltrating inflammatory cells cannot be ruled out by this data; however, HA was also abundant in the stroma when only little infiltrate was present (data not shown). Indeed, we show that *in vitro* Fbs outperform MM in regard to HA synthesis by more than the factor 1,000. These data are supported by comparative mRNA analyses of the HA-synthesizing enzyme HAS2, which is significantly stronger expressed in Fbs than in MM. In accordance with previous data (Gebhardt *et al.*, 2010), the two other known HASs HAS1 and HAS3 are only expressed at low mRNA levels in both cell types. These findings suggest that the majority of tumor-promoting stromal HA originates from Fbs. Likewise, in breast cancer stromal Fbs are considered to be responsible for HA accumulation (Itano *et al.*, 2008). In breast and prostate cancer, HA amount and tumor aggressiveness correlate (Auvinen *et al.*, 2000; Lipponen *et al.*, 2001). Stromal HA enhances tumor cell invasion and motility (Afify *et al.*, 2009) and HA diminishes intercellular adhesion, thereby promoting cell migration (Itano *et al.*, 2002). HA has a pivotal role for proliferation in different tumors (Ahrens *et al.*, 2001; Edward *et al.*, 2005; Misra *et al.*, 2008). Our own experiments confirmed an enhanced MM proliferation depending on Fbs' HA synthesis. The silencing of HAS2 in Fbs strongly decreased their HA synthesis, resulting in decreased MM cell proliferation. Decreased HAS2 expression in silenced Fbs was not compensated by an induction of HAS1 or HAS3 (data not shown). Pre-treatment of Fb-CM with hyaluronidase removed the detectable HA, and consequently the induction of MM proliferation by these Fb-CM also decreased (data not shown). Nevertheless, the low synthesis of tumor-cell–associated HA also provides crucial benefits for the tumor itself, as shown by gain-of-function (Ichikawa *et al.*, 1999; Kosaki *et al.*, 1999) or loss-of-function (Simpson *et al.*, 2002; Udabage *et al.*, 2005; Li *et al.*, 2007b) studies in several tumor models. CD44 was shown to be the receptor on tumor cells that is involved in the HA-mediated induction of cell proliferation (Ahrens *et al.*, 2001; Morrison *et al.*, 2001; Anderegg *et al.*, 2009). Indeed, only the proliferation of CD44 expressing cell lines *Bro* and *HT144* was increased by HA-containing Fb-CM, whereas the proliferation of CD44-negative MM cell line *RPM-MC* ((Hoashi *et al.*, 2001; Morrison *et al.*, 2001; Anderegg *et al.*, 2009) remained unchanged (data not shown).

MMs are able to elevate HA synthesis in Fbs, which has been shown before (Knudson *et al.*, 1984; Merrilees and Finlay, 1985; Godden *et al.*, 1999; Edward, 2001).

The stimulation of HA synthesis by MM-CM can depend on the glucose content in the medium; specifically, low glucose levels (1.0 mg ml^{-1}) in MCCM were superior in inducing HA (Edward *et al.*, 2005). The MM-CM used here contained 0.38 ± 0.13 mg ml^{-1} glucose when they were transferred to Fbs. Low glucose levels have been shown not to affect the HA release of Fbs during short-term incubations, similar to those used in this study (Cechowska-Pasko and Bankowski, 2010). Thus, under low glucose conditions used

here only the growth factors within the MM-CM are expected to modify HA synthesis of Fbs.

We found induction of HAS1– and HAS2–mRNA transcription in Fbs exposed to *Bro*-CM and *HT144*-CM, but not *A375*-CM. This enabled us to identify the soluble factors from MM involved in HA induction in Fbs. Lactate, previously described as an inductor of HA synthesis (Stern *et al.*, 2002), was excluded, as lactate concentrations in MM-CM did not correlate with their HA-stimulating potential, which was however trypsin-sensitive indicative of a protein mediator.

Indeed, HA synthesis can be induced by proteins such as IL-1β, TNFα (Campo *et al.*, 2006), TGF-β_1 (Meran *et al.*, 2007), or PDGF-BB (Li *et al.*, 2007a) via induction of transcription of HASs. However, the effectiveness of the cytokines may differ in various cell types (Kennedy *et al.*, 2000; Ducale *et al.*, 2005; Meran *et al.*, 2007). Additionally, HAS activity may also be regulated by posttranslational modifications such as phosphorylation (Vigetti *et al.*, 2009) and ubiquitination (Karousou *et al.*, 2010).

In our experiments, recombinant TGF-β_1 increased HA synthesis in dermal Fbs by induction of HAS1– and HAS2–mRNA. TGF-β_1 concentrations in MM-CM matched the HA-stimulatory capacity of MM, so we initially focussed on MM-derived TGF-β_1 as potential mediator. When silencing TGF-β_1 expression in MM, the resulting TGF-β_1-reduced MM-CM failed to induce HAS1–mRNA expression, whereas HAS2–mRNA expression was differently affected in the inducing MM cell lines. Obviously, TGF-β_1 has a predominant influence on HAS1 expression as reported earlier (Stuhlmeier and Pollaschek, 2004). However, net HA release by Fbs did not decrease. The major HA-synthesizing enzyme in Fbs is HAS2 (Averbeck *et al.*, 2007), and although TGF-β_1 cannot be completely ruled out as a HAS2–mRNA inductor referring to earlier reports (Heldin *et al.*, 1989; Sugiyama *et al.*, 1998), other MM-derived factors seem to compensate a lack of TGF-β_1. These findings are in line with the study by Godden *et al.* (1999) when blocking TGF-β with an antibody also was not effective in reducing induction of HA synthesis. Blocking experiments revealed the involvement of PI3/MAP-kinase signaling in HAS2 induction in Fbs.

Various growth factors function through PI3/MAP-kinase pathway, including PDGF. Godden *et al.* (1999) demonstrated that PDGFR-α was phosphorylated in Fbs after exposure to MM-CM, and proposed PDGF-AA, PDGF-AB, and PDGF-BB as key inductors of the increased HA synthesis. We could confirm this for PDGF-AA, but could not demonstrate a role for PDGF-AB and PDGF-BB. When comparing growth factor release from strongly HA-inducing *Bro*-MM and weakly HA-inducing *A375*-MM, PDGF-AA was identified as the most probable factor to induce HAS2 in Fbs. We also suspected PDGF-CC, a more recently discovered member of the PDGF family (Li and Eriksson, 2003), to be involved. PDGF-CC was reported to be an MM-derived stimulator of osteopontin in stromal Fbs (Anderberg *et al.*, 2009). We found by mRNA analysis that expression patterns of PDGF-A and PDGF-C matched the HA-inducing properties of the respective cell lines, while PDGF-B was barely detectable in all MM.

However, individual silencing of PDGF-A– or PDGF-C–mRNA in *Bro*- and *HT144*-MM had no effect on HA synthesis in Fbs. Interestingly, PDGF-AA and PDGF-CC seem to compensate for each other, as silencing of one mRNA led to significantly increased expression of the other. Double knockdown of PDGF-A/PDGF-C in MM clearly reduced HAS2–mRNA induction, but reduced HA synthesis by Fbs at best by 30%, which is most likely to residual PDGF-AA or PDGF-CC. The remaining concentrations of PDGF-AA measured in MM-CM have been shown earlier to contribute to induced HA synthesis in Fbs (Heldin *et al.*, 1989).

As another approach to test the role for melanoma-derived PDGF-AA and PDGF-CC in inducing HA release from Fbs, we blocked PDGFR-α—the receptor for both PDGF-AA and PDGF-CC (Fredriksson *et al.*, 2004; Anderberg *et al.*, 2009). Indeed, pre-incubation of Fbs with neutralizing antibodies against PDGFR-α reduced HAS2–mRNA induction as well as significantly diminished HA synthesis in Fbs.

This confirmed PDGF-AA and PDGF-CC as key MM-derived factors to induce HA synthesis via HAS2 induction in Fbs. As HAS1 was not affected by blocking PDGFR-α signaling, we conclude that HAS2 is the main target of MM-cell–derived PDGF-AA and PDGF-CC. Although PDGF-Rα blocking reduced HA production by more than 50%, we expect that other MM-derived soluble factors might function synergistically with PDGF-AA and PDGF-CC in stimulating HA synthesis in Fbs.

Taken together, we demonstrated that MM are able to induce HA synthesis in pertitumoral dermal Fbs via induction of HAS2. This is controlled by the release of soluble proteins from MM, especially PDGF-AA and PDGF-CC. HA synthesized by dermal Fbs in turn stimulates MM proliferation. This supports the notion that in MM the interaction of tumor cells with stromal Fbs regulates local HA metabolism, which is of role in tumor progression.

MATERIALS AND METHODS

Cell culture

Primary Fb cultures obtained by outgrowth from skin biopsies and melanoma cell lines *Bro* (kindly provided by Dr J Eberle Berlin; Lockshin *et al.* (1985)), *HT144* (kindly provided by Dr van Muijen; van Muijen *et al.* (1995)) and *A375* (ATCC-CRL1619) were cultured in 1:1 mix of RPMI-1640 and DMEM (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (PAA, Pasching, Austria), Penicillin/Streptomycin (Biochrom) and Mycokill (PAA). All cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. To generate CM, cells were washed with phosphate-buffered saline and incubated with serum-free 1:1 mix of RPMI-1640 and DMEM for 24 hours. The medium contained 1.5 mg ml^{-1} glucose. CM was harvested, centrifuged and stored at −80 °C until use. The CM was not concentrated, used without additional nutrition and contained 0.38 ± 0.13 mg ml^{-1} glucose. Cell numbers were determined using the Countess device (Invitrogen, Karlsruhe, Germany). Before experiments, MM and Fbs between passages 3–6 were washed with phosphate-buffered saline and maintained in serum-free 1:1 mix of RPMI-1640 and DMEM to synchronize cell cycle over night. Medium transfer experiments were performed by exposing Fbs or MM monolayer cultures to CM for 3 hours to analyze mRNA or

24 hours for HA measurements. Fbs were stimulated with 10 ng ml^{-1} TGF-β_1 (Strathman Biotech, Hamburg, Germany) for 3 hours to analyze mRNA or for 24 hours for HA measurements.

Proliferation assays

Serum-starved MM cells (1.6×10^4 in 150 µl DMEM with 4% fetal calf serum) were mixed with 500 µl DMEM (4% fetal calf serum) or with 500 µl Fb-CM and splitted in 4 wells (150 µl each) of black 96-well plates (Nunc, Roskilde, Denmark). After 20 hours, BrdU was added for 4 hours and proliferation was measured by BrdU-chemiluminescence ELISA (Roche, Mannheim, Germany), according to the manufacturer's protocol.

SiRNA transfection of MM

SiRNA (Stealth Select RNAi) specific for TGF-β_1 (Stealth Select RNAi HSS110683 and HSS110684) and scrambled siRNA (Stealth RNAi Negative Control (High and Medium GC) were purchased from Invitrogen. MMs were transfected with either 40 pmol ml^{-1} gene-specific siRNA or 40 pmol ml^{-1} scrambled control siRNA using Lipofectamine (RNAiMax, Invitrogen), according to the manufacturer's instructions. After 48 hours, TGF-β_1–mRNA was quantified by RT-PCR. TGF-β_1 concentrations in MM-CM were measured by ELISA (R&D Systems, Minneapolis, MN). SiRNA specific against PDGF-A (ON-TARGETplusSMARTpool L-013154-01-0010), PDGF-C (ON-TARGETplusSMARTpool L-011748-00-0010), and negative control (On-TARGETplus Non-Target; Dharmacon, Lafayette, CO) were purchased from Fisher Scientific (Schwerte, Germany). Transfection was performed as described above. PDGF-A– and PDGF-C–mRNA were measured after 72 hours by quantitative RT-PCR. Silencing of MM was achieved without eliciting critical stress–responses confirmed by quantitative RT-PCR of the stress gene 2′–5′ oligoadenylate synthetase (Sledz *et al.*, 2003).

SiRNA transfection of Fbs

SiRNA specific for HAS2 and HAS2, as well as scrambled siRNA were purchased from Ribbox (Radebeul, Germany). Sequences are provided in Supplementary Table S1 online. 2.25×10^4 Fbs per well were transfected with either 5–20 nM of gene-specific siRNA or 5–20 nM of scrambled control siRNA using ribboxxFECT (Ribbox), according to the manufacturer's instructions. After 36 hours, transfection medium was replaced by DMEM containing 4% fetal calf serum (0.6 ml per well of a 24-well plate). This medium was harvested after 24 hours of incubation. At this time point, HA content was determined and these Fb-CMs were used for cell proliferation assays. Fbs were used for quantitation of HAS mRNA.

Blocking experiments

MM-CM was incubated with immobilized Trypsin (Pierce, Rockford) according to the manufacturer's instructions. Fbs were incubated in parallel with MM-CM or trypsin-treated MM-CM for 3 hours.

Inhibition of PI3/MAP-kinase pathway was performed using MAPK/ERK kinase 1/2 10 µM inhibitor U0126 and PI3K inhibitor LY294002 (both Calbiochem, Darmstadt, Germany) by simultaneously incubating Fbs with MM-CM, MM-CM + U0126, or MM-CM + 20 µM LY294002 for 3 hours and 24 hours. Fbs were exposed to CM of siRNA-transfected MM for 3 hours or 6 hours to analyze mRNAs and 24 hours to determine HA concentrations.

Neutralization experiments were performed by pre-incubating Fbs with 10 µg ml^{-1} anti-PDGFR-α (R&D Systems) or normal mouse-IgG (isotype control, R&D Systems) before exposure to MM-CM for 3 hours and 24 hours.

RNA preparation and complementary DNA synthesis

RNA preparation and complementary DNA synthesis were performed as described (Anderegg *et al.*, 2009). Primer pairs for quantitative RT-PCR were designed using HUSAR software (EMBL, Heidelberg, Germany) and are indicated in Supplementary Table S1 online. Quantitative RT-PCR was performed as described (Anderegg *et al.*, 2009; Gebhardt *et al.*, 2010).

Immunoaffinity staining for HA

The study was conducted according to the Declaration of Helsinki Principles.

HA staining of MM biopsy for HA with biotinylated HAbP (Seikagaku, Tokyo, Japan) including the pre-treatment of sections with hyaluronidase for control of specific HAbP binding were performed as described (Averbeck *et al.*, 2007).

Second, double stainings were performed with HAbP (Seikagaku) and the anti-CD90 antibody AS02, which detects Fbs and activated microvascular endothelium (Saalbach *et al.*, 1997). HAbP was visualized with Streptavidin-488 (Mobitec, Goettingen, Germany) and bound CD90 was detected by goat-anti mouse-Cy3 (Dianova, Hamburg, Germany).

HA-ELISA

HA concentrations were measured by ELISA (Corgenix, Broomfield, CO) following the manufacturer's protocol. Samples were assayed as duplicates and cell numbers were determined after sampling. All HA concentrations were normalized to cell counts.

Growth factor array and ELISA

Expression of growth factors in *A375*- and *Bro*-CM was analyzed using RayBio Human Growth Factor Antibody Array 1 Map (RayBiotech, Norcross, GA) according to the manufacturer's instruction. Data from two different MM-CM were used from each cell line. PDGF-AA was measured by ELISA (Raybiotech) according to the manufacturers' recommendations.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed with GraphPad Prism 4 (GraphPad Software, La Jolla, CA) using unpaired *t*-test, paired *t*-test, or Wilcoxon sign-rank test.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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